Studies on the broad spectrum bioactivity of probiotic bacterium *Lactobacillus delbrueckii*

Abstract:

Human vagina contains *lactobacilli* that are the part of vaginal microbiota. The identification and assessment of the unique properties of usual *Lactobacillus species* in the vagina of healthy women will assist in selecting the best species. In the current study we have carried out the isolation and identification of *Lactobacillus species* from the vagina of healthy women to explore and evaluate their probiotic and antimicrobial efficacy. The *Lactobacillus* was identified by the phenotypic and biochemical tests. *Lactobacillus species* were identified by molecular method by amplification of the full length of the 16S rDNA of the isolated bacteria using polymerase chain reaction. In this study, eight vaginal lactic acid bacillus isolates manifested plethora of in vitro probiotic characteristics and showed potential activity against the most prevalent bacterial pathogens. Bacteriocin was isolated from *Lactobacillus delbrueckii* and showed antimicrobial properties. MALDI-TOF investigation was performed with the crude supernatant acquired from an initial stationary phase culture in the MRS broth. Bacteriocin was proteolytic enzyme sensitive, heat resistant, functional over a broad pH range, and drifted as a low molecular weight peptide in the SDS–PAGE.

1.0 Introduction

The vaginal ecosystem's homeostasis gets benefited by the complex interactions and synergies between the host and various microbiota that colonize the mucosa of the vagina. *Lactobacillus spp* is typically the dominant bacterial flora found in the healthy vaginal microbiota (Petrova *et al.,* 2015). By developing antimicrobial compounds, or by competition for the attachment to the epithelium of vagina, these bacteria exhibits pivotal defence mechanism against deadliest pathogens. There is a growing interest in their use of probiotic formulations for the prophylaxis and treatment of many vaginal infections because of the favourable effects of *lactobacilli* on the health of the women's genital tract (Borges *et al.,* 2014).In the last decade lactic acid bacillus, along with bifidobacteria, have been the most commonly examined probiotics. It is important to test these probiotic microorganisms for the existence of probiotic properties that include antimicrobial efficacy against specific pathogens and the development of anti-microbial compounds (Parvez *et al.,* 2006). Recently, several attempts are made to search for possible human-isolated probiotic strains of *Lactobacillus* (Dover *et al.,* 2008).In the current study the isolation and identification of *Lactobacillus* species from the vagina of healthy women to explore and evaluate their probiotic and antimicrobial efficacy was carried out.

2.0. Materials and methods

2.1. Sampling and study area

In this cross-sectional experimental study, samples were collected at Melmaruvathur Adhiparasakthi Institute of Medical Sciences and Research, Tamilnadu, and the collected samples were processed at Central Research Laboratory, Meenakshi Academy of Higher Education and Research (MAHER), Chennai. The study was approved by the

Institutional ethical committee. The study was conducted from January 2015 to December 2018 in pre-menopausal Indian women (15-45 years) who did not have any symptoms of vaginal or urinary tract infection. A systematic proforma was obtained to get details from patients who pursued with Clinical examination. Subjects were excluded if they have the consequences such as history of hysterectomy, evidence of vulval, genital or cervical dysplasia over the last5 years, history of candidiasis, urinary tract infections or active sexually transmitted disease, history of regular urinary incontinence**,** menstruation, contraceptives and intrauterine devices (Parolin *et al.,* 2015; Pascual *et al.,* 2008).

2.2. Collection of vaginal swab for the isolation of vaginal microbiota

Three specimens were collected from each participant. Sample was collected from the vaginal posterior fornix, introitus, and mid-vagina under strict aseptic conditions. Precautions were taken during sample collection to avoid affliction in the region where the speculum touched (Stoyancheva *et al.,* 2014; Antonio *et al.,* 1999)

2.3. Microscopy of the specimen

2.3.1. Grams staining

The specimen from the swab was used for smear preparation. Contents present on the swab were transferred to a clean grease free slide by rolling the swab on the slide. Precautions were taken to spread the collected material on the slide without interrupting the morphology of bacteria and other structures. Smears were fixed by air drying and Grams' staining was performed (Bartholomew and Mittwer, 1952).

2.3.2. Nugent's scoring of the isolates

The score of Nugent's grading is based on the classification of value ranges between 4-6 in the presence of clue cells or >7 are considered bacterial vaginosis, while scores from 0 to 3 and 4 to 6 (without clue cells) are considered normal and intermediate, respectively.

The value is obtained by the addition of three different scores; in turn, every score represents the quantity of a specific bacterial morphotype which can be evaluated through the optical microscope (Burton and Reid, 2002; Delaney *et al.,* 2001).

2.4. Isolation of bacteria from the swabs

Rogosa sheep blood agar medium was used as a culture medium for the growth of *Lactobacilli* from vaginal swabs. The culture plates were incubated at 37^{o}C for the time period of 24–48 h with 5% CO_2 in an anaerobic jar containing Anaero Pack (Himedia). Plates with visible characteristic growth were further studied. The colonies with Gram-positive rods were inoculated in the culture medium containing de Man-Rogosa-Sharpe (MRS) broth. Individual colonies were grown in2.5 ml of MRS broth incubated overnight in 5 % CO_2 at 37^{o}C. Glycerol (10 %) stocks were prepared in 200 µL of the broth culture and stored at -80°C. One set of swab was kept in Blood agar (Himedia) and Sabraud's dextrose agar (Himedia) which was used to culture facultative aerobic bacteria and fungi respectively from a vaginal swab (Martinez-Pena *et al.,* 2013; Hillier *et al.,* 1992).

2.5. Physiological and biochemical characteristics for identification of *Lactobacillus*

2.5.1. Determination of sugar fermentation

The fermentation assays for sugars were carried out using 1 percent (w/v) sugar in the MRS broth. In this test, Glucose, Fructose, Sucrose, Xylose and Lactose were used and 0.01 % Phenol Red was used as an indicator solution. 10 ml of media was distributed in each of the test tubes and the Durham tubes were inserted inverted. The culture (overnight inoculated culture) was inoculated and incubated for 24 hours at 37 ° C. An un-inoculated sterile media was maintained as a negative control. Results were noted according to the colour change and gas formation (Sanchez *et al.,* 2000).

2.5.2. Biochemical parameters

The isolated pure strains were streaked on MRS culture medium and incubated at 37°C for 24 hrs. Oxidase test, Catalase test, Gelatin liquefaction test, Nitrate reduction test, litmus milk test, hydrogen sulfide test, Methyl Red test, V.P. test, Indole test, growth temperature test (10°C, 15°C, 45°C, 60°C for 30 min), 6.5% NaCl growth test, pH gradient test and sugar alcohol fermentation test were performed (Cai *et al.,* 1998).

2.6. Molecular characterization by 16srRNA sequencing

Using the CTAB-NaCl process, the genomic DNA was extracted. PCR analysis was performed using the 27F (5-AGA GTT TGA TCM TGG CTC AG-3), 1492R (5-CGG TTA CCT TGTTAC GAC TT-3) primer combination for 16S rRNA gene amplification. NCBI BLASTn (https:/blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare the 16S rRNA gene sequence obtained from the isolates and ClustalW 1.83 (https:/www.ebi.ac.uk/cgi-bin/clustalw) performed multiple sequence alignment. Phylogenetic trees were built by neighbour joining (NJ) and minimum evolution (ME) in MEGA 6.0 (Jandaand Abbott, 2007).

2.7. Multiplex PCR assay for the determination of *Lactobacillus sps.*

A multiplex PCR assays were carried out with the primers which are listed in Table 1. PCR master mix (50 µl) which contains 0.25mM of primers, 1× Taq DNA polymerase buffer, 50ng of genomic DNA, 0.2 mM of each dNTP, 1 U of Taq DNA polymerase, and 1.5 mM$MgCl_2$ was used. PCR was employed with an initial denaturation for 3 min at 94 °C, 30cycles of denaturation for 30 s at 94 °C, annealing for 10 s at 56 °C, and elongation for 30 s at72 °C, and a final extension for 5 min at 72 °C. PCR products were observed in 1.5% agarose gel after electrophoresis and the scale was compared with a 100 bp ladder (Wilson, 2001).

Table 1 - Species identification by multiplex PCR

S. No	Program	Species	Amplicon	Primer (5'-3' sequence)
1	PCR II-1	*L. acidophilus*	210 bp	L-aci-1 (TGCAAAGT GGTAGCGTAAGC) 23-10C CCTTTCCCTCACGGTACTG
2	PCR II-2	*L. crispatus*	522 bp	Lcri-3 AGGATATGGAGAGCAGGAAT Lcri-2 CAACTATCTCTCTTACACTGCC
		L. gasseri	360 bp	Lgas-3 AGCGACCGAGAAGAGAGAGA Lgas-2 TGCTATCGCTTCAAGTGCTT
3	PCR-III	*L. paracasei*	312 bp	Lpar-4 GGCCAGCTATGTATTCACTGA
		L. rhamnosus	113 bp	RhaII GCGATGCGAATTTCTATTATT
		L. salivarius	411 bp	Lsal-1AATCGCTAAACTCATAACCT Lsal-2 CACTCTCTTTGGCTAATCTT
4	PCR-IV	*L. reuteri*	303 bp	Lreu-1 CAGACAATCTTTGATTGTTTAG Lreu-4 GCTTGTTGGTTTGGGCTCTTC
		L. plantarum	355 bp	Lpla-3 ATTCATAGTCTAGTTGGAGGT Lpla-2 CCTGAACTGAGAGAATTTGA
		L. fermentum	192 bp	Lfer-3 ACTAACTTGACTGATCTACGA Lfer-4 TTCACTGCTCAAGTAATCATC
		L. delbruckii	1065 bp	Ldel-1 5'AAGGAGGTGATCCAGCCGCA3'

2.8. Probiotic attributes of *Lactobacillus* isolates form vaginal environment

2.8.1. Acid and bile salt tolerance of the *Lactobacillus* isolates

MRS broth was used to determine the tolerance of the bacterial strains to acid (pH 3.0) and bile salt (0.3 percent w/v) were investigated. 1.5 ml of NaCl (0.5 w/v) and 5 ml of simulated gastric, 1 ml of washed *Lactobacillus* cell suspension was incubated for 3 hours. Simulated gastric juice was freshly prepared by suspending pepsin (3 g/L) in sterile saline (0.5 per cent w/v) and the pH was adjusted to 2.0 with concentrated HCl. Aliquots of 0.1ml were then extracted to assess the total viable count. Dilutions were made (up to 10^{-4}) and the cells were duplicated on MRS agar. Plates were incubated for 72 h at 37 °C before counting (Martin Rosique *et al.,* 2008).

The MRS broth containing bile was prepared by adding 0.3 (v/v) of bile salt. Cells were then collected by centrifugation from 100 ml (20 h MRS tested culture) (3400 RPM, 10 min), washed two times in saline (8.5 g NaCl/L) and resuspended in 10 ml MRS broth. This suspension was inoculated into MRS broth (1%) that lacked or contained bile salt. After incubation at 37 °C for 0, 1, 2 and 3 h, viable counts on MRS agar plates and culture absorption at 625 nm was measured (Jang *et al.,* 2019). Acid and bile tolerance assay was performed thrice with duplicates.

2.8.2. Hydrogen peroxide production

Lactobacillus sps., cells were resuspended with 55.5 mM glucose in a cold phosphate buffer (0.2M, pH 6.5) and incubated at 5°C for the desired period. Cells were extracted after incubation by centrifugation and the supernatant was used for the test of hydrogen peroxide level. The hydrogen peroxide production assay was performed with the culture supernatant in a test tube containing 1 ml of 0.1 percent aqueous peroxidase solution (Horseradish Type VI-A; Sigma Chemical) and 100 µl of 1percent aqueous o-dianisidine solution (Sigma Chemical), respectively. Instead of a test sample, 5 ml of sodium

phosphate buffer was taken as blank. Tubes were incubated at 37°C for 10 min. The reaction was stopped by adding 200 µl of 4N HCl to each test tube. Absorbance (A400 nm) was taken for each sample and the peroxide amount was calculated by comparing the A_{400} nm absorbance with the standard curve (Chetwin *et al.,* 2019).

2.9. Determination of antimicrobial potential of the *lactobacillus* isolates

The antibacterial activity of the *lactobacillus* isolates was analyzed by well diffusion assay. In soft nutrient agar of 20 ml (0.8 percent, w/v), 100 µl of early stationary phase test bacterial culture was added. On the lawn of hardened soft agar in Petri dishes, Wells were bored. Aliquots (100 µl) of the overnight culture supernatant (16-18 h) were poured into the wells. The plates were left in sterile conditions at room temperature for 1 hour, and then incubated for the growth of the test micro-organism at 37 ^{o}C. A clear zone (at least 2mm in diameter) of inhibition was considered as potential antimicrobial activity (Pithva *et al.,* 2014).

3.0. Results

3.1. Identification of *Lactobacillus*

The Gram's staining results, depicts that the isolates showed clear bacilli structure. Most of the isolates which were Catalase negative were also negative for citrate utilization and Indole test.

3.2. Nugent's score of the isolates

Gram stained smears of all the women were scrutinized using Nugent's scoring system. Majority, 82.7% of the women had normal vaginal flora corresponding to a score between 0-3.

3.3. Biochemical characteristics for identification of *Lactobacillus*

Among various sugars examined both isolates fermented the following sugars that include Glucose, Fructose, Sucrose, Mannitol, Maltose, Mannose, Galactose, D-Xylose,

Trehalose, Salicin and Cellobiose, but they failed to ferment D-Arabinose, Rhamnose, Sorbitol and Starch. The isolates were identified as members of lactic acid bacteria based on the sugar fermentation results.

3.4. Molecular characterization by 16s rRNA sequencing

3.4.1. 16s rRNA sequencing

16S loci in 8 different species of *Lactobacillus* were amplified successfully. A concentration of 2.5mM of MgCl2, 2.5mM of dNTP, 20 µM of primer, 3 units of Taq DNA polymerase and 30ng/µl of template DNA was found to be optimal for the amplification. Optimum annealing temperature for each primer was standardized by gradient PCR. Using universal primers 27F and 1492R, the 16S rRNA gene of the isolates was amplified. A single band with a length of about 1500 bp was obtained for all amplified items.

3.4.2. Sequence alignment of *Lactobacillus spp.*

To check the homology among the isolates and with others, the sequences were scrutinised by multiple sequence alignment. The similarity between the sequences varied from 91 to 98% among the isolates. Minimum of 91% homology was found in 15P1B1 with *Lactobacillus delbrueckii* and a maximum of 98% similarity was found between 22P5B2 and *L. reuteri*. None of these isolates were shown 99% or more similarity among themselves.

3.4.3 Identification of *lactobacilli* at the species level using multiplex PCR assays

Using multiplex PCR, *L. salivarius*, *L. delbrueckii, L. reuteri*, *L. plantarum*, *L. crispatus*, *L. fermentum*, *L. rhamnosus*, and *L. acidophilus* were successfully characterized by one of the four second-step multiplex PCR assays at the species level. The specificity of each group- and species-specific primer pair was confirmed using PCR amplification

with DNA samples of 8 *Lactobacillus* prior to the demonstration of the multiplex PCR assays.

3.5. Probiotic attributes of *Lactobacillus spp.*

3.5.1. Microbial adhesion to solvents

The use of three solvents was to test the hydrophobic/hydrophilic cell surface properties of *Lactobacilli* and their Lewis acid-base (electron donor and acceptor) properties. There is also an acidic surface character of these bacteria. *L. crispatus* strains also exhibited relatively good adherence value (35%) to ethyl acetate.

3.5.2. Hydrogen peroxide production by the isolated *Lactobacillus Sps.*

Hydrogen peroxide is another antagonistic compound produced by *Lactobacilli*. Generally its production is evaluated using quantitative methods. In the current study, the isolate *Lactobacillus delbrueckii* showed maximum H_2O_2 production i.e. 3.18 mg/ml and minimum 0.31mg/ml was observed in *L. acidophilus*, while all other isolates exhibited moderate production of H_2O_2 ranging from 0.4-0.86 mg/ml.

3.5.3. Survival of *Lactobacillus* strains under acidic condition

The maximum acidity of the stomach and the regions following the stomach and the pH of these areas can decrease to pH 2.0. *Lactobacillus* could be able to endure these hard environments and colonize the gut and thus, resistant acidity so that it can be used as a beneficial adjunct.

3.6. Auto-aggregation of *Lactobacillus* strains

Based on the sedimentation features of the strains, auto-aggregation was analysed because when cells accumulate, they clear the supernatant and sediment.

3.7. Antimicrobial activity of the isolates

The preliminary efficiency of the antimicrobial activity of the strains was evaluated by the agar well diffusion method.

4.0. Eventually, *L. delbrueckii* was chosen for further tests of characterization and antibacterial activity, because it has significantly higher antibacterial activities when compared to other strains ($p < 0.05$). Among the isolates, *Lactobacillus delbrueckii* exhibits potential antimicrobial activity against most of the tested pathogens, hence it was fixed as an organism of interest with great antimicrobial potential. Hence, an attempt was made in this study on production, purification and characterization of bacteriocin from human vaginal bacterium *Lactobacillus delbruckii*.

4.1. Determination of Bacteriocin Activity: Bacteriocin activity was determined by the agar well diffusion assay (AWDA). Wells with a diameter of 6 mm was bored on the Mueller-Hinton agar plates which are seeded with the pathogenic organism and the aliquots (20 µl) of the sterile supernatant were added in the well and incubated for 24 hours at temperature of 37^0C. After 24 hours of incubation, the growth inhibition diameter was measured. The activity of the cell-free supernatant was expressed in activity units (AU). A unit activity of the bacteriocin was defined as activity unit; 1 AU is a unit area of inhibition zone per unit volume, in this case, mm^2/ml. The assay was performed in triplicate (Usmiati and Marwati, 2009).

The bacteriocin activity was calculated using the following formula:

$$\text{Bacteriocin activity } (mm^2/ml) = \frac{Lz - Ls}{V}$$

L_z = clear zone area (mm^2)

L_s = well area (mm^2)

V = volume of sample (ml)

4.2. Partial characterization of bacteriocin: Before the partial characterization of bacteriocin, the bacteriocin-producing *L. delbrueckii* isolate was grown in MRS broth for 48 h at 37 ºC. After incubation, the cultures were centrifuged at 10,000 rpm for 60 minutes at 4ºC, the bacteriocins were precipitated with (80% saturation) ammonium sulfate and kept overnight at -20ºC for enhanced precipitation. Following precipitation, centrifugation was done and the pellets were collected and stored in a phosphate buffer with a pH of 5.5. The precipitate was dialyzed against 20 mM potassium phosphate buffer (pH 7.0) 52 for 12 h at 4ºC (Rajaram *et al.,* 2010). All the experiments were performed in triplicate and standard error was found to be within 5%.

5.1. Effect of Temperature on Bacteriocin Activity: The dialyzed bacteriocin (pH 5.5, 500 µl) was exposed to various heat treatments: 30°C, 40°C, 60°C, 80°C and, 100°C for 30 minutes and 121°C for 15 minutes. Bacteriocin activity was determined against Bacillus cereus as an indicator organism (Rajaram *et al.,* 2010).

5.2. Effect of pH on Bacteriocin Activity: The effect of pH was tested with the bacteriocin (pH 5.5, 50 µl) which was mixed with 100 µl of buffer (0.1M potassium phosphate pH4, pH5, pH6, pH7, pH8, and pH9) and incubated for 1 hour at 60°C, and then the bacteriocin activity against the organism *Bacillus cereus* was determined (Rajaram *et al.,* 2010).

5.3. Effect of detergents on Bacteriocin Activity: Triton-X100, Tween 80, sodium dodecyl sulfate, and Tween 80 surfactants were added in the concentration of 0.1 ml or 0.01 g/ml to the bacteriocin solutions (pH 5.5). These preparations were incubated at 60°C for 60 minutes and bacteriocin activity against the indicator strain *Bacillus cereus* was determined (Rajaram *et al.,* 2010).

5.4. Effect of Enzymes on Bacteriocin Activity: The following enzymes D-amylase, lysozyme, protease, and proteinase-K each with a final concentration of 1 mg/ml were tested on bacteriocin preparation (pH 5.5). It was then incubated at 60°C for 2 hours, and bacteriocin activity against the pathogens was determined (Rajaram *et al.,* 2010).

6.0. Bacteriocin activity determination and calculation: Activity units (AU) of bacteriocin activities were calculated according to the spot-on-lawn method (Yamamoto *et al.,* 2003).

7.0. Passive Elution of bacteriocin from Polyacrylamide Gel: *Lactobacillus delbrueckii* crude resulting proteins were fractionated by Tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (16% polyacrylamide gel) using the method of Schagger and Von Jagow., 1987. After completion of electrophoresis, each gel was stained with Coomassie brilliant blue. To detect protein bands with antibacterial activity, the gel was washed three times in 0.1% Tween 80 (40 min each) at room temperature to remove SDS. Then soft nutrient agar medium (10 ml) containing test pathogens (approximately $1x10^6$ to $2x10^6$ cells/ml) was overlaid to cover the whole gel (6 by 9 cm) using the method of Martinez *et al.,* 2013. Clear zones around the protein bands were observed after incubation at 30°C overnight which delineates the antibacterial activity of the protein.

8.0. Identification of Bacteriocin by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry: Entire mass spectra were acquired on a linear MALDI-TOF mass spectrometer equipped with delayed extraction technology.

9.0. Bio assays of bacteriocin

9.1. Antifungal effects of *L. Delbrueckii* bacteriocin in vitro: The microdilution method described by Lavermicocca *et al*., 2003 was used to test the efficiency of *L. delbrueckii* bacteriocin against fungal pathogens at 5 and 20°C. Conidia of the fungi were collected from 12 day old cultures grown under a 12 h light/dark cycle. The density of spore suspensions was diluted using sterile distilled water, to 2×10^5 spore's ml^{-1} as determined using a haemocytometer (Hausser Scientific, PA, USA). Micro dilution tests were performed in sterile 96-well microdilution plates. 200 µl of test solution which contains 185 µl of *L. delbrueckii* bacteriocin and 15 µl of conidial suspension was dispensed into the wells, after that the microdilution plates were incubated. The optical density (OD) was recorded in specific time intervals at 580 nm by using a microtiter plate reader/ spectrophotometer. (Spectra MAX 190 Molecular Devices, CA, USA). The experiment was done in triplicates.

9.2. Anti-cancer activity of bacteriocin: The anticancer activity of *L. delbrueckii* bacteriocin was evaluated using five different human cancer cell line which includes HeLa, MCF-7, HT1080, H1299, and HEK293T. The cell lines were incubated with bacteriocin with increasing concentration (1–20 µM) in their respective growth medium. Dose-dependent cytotoxicity was obtained in all cancer cell lines (Ankaiah *et al.,* 2018).

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enterocin-A, B against pathogenic bacteria and human cancer cells. *International Journal of Biological Macromolecules*, **116**, 502-512.

Abbreviations:

AU	- Activity units
AWDA	- Agar well diffusion assay
BLAST	- Basic Local Alignment Search Tool
bp	- Base pairs
CO2	- Carbon dioxide
CTAB	- N-cetyl N,N,N trimethyl ammonium bromide
DNA	- Deoxyribonucleic Acid
H_2O_2	- Hydrogen peroxide
HCl	- Hydrochloric acid
MALDI TOF	- Matrix Assisted Laser Desorption/Ionization Time-of-Flight
$MgCl_2$	- Magnesium Chloride
MRS broth	- de Man Rogosa Sharpe
NaCl	- Sodium Chloride
NCBI	- National Centre for Biotechnology Information
OD	- Optical density
PAGE	- Polyacrylamide Gel Electrophoresis
RNA	- Ribonucleic acid
RPM	- Revolutions per minute
SDS	- Sodium Dodecyl Sulfate
V.P. test	- Voges Proskauer test

LIST OF PUBLICATIONS

1. Vijaya, R., Revathi, K., Arunagirinathan, N., Senthil Pragash, D. and Khaja Mohiddin, S.2020. Exploration of probiotic attributes of *Lactobacillus Sps*. from vagina and its rapid identification by multiplex PCR assays using group specific primers. *European Journal of Molecular & Clinical Medicine*,**7**, 4171-4189.

2. Vijaya, R., Revathi, K., Arunagirinathan, N., Senthil Pragash, D. and Khaja Mohiddin, S. 2021.Isolation, characterization of *Lactobacillus delbrueckii* derived bacteriocin and assessment of its bioactive potential. *Annals of R.S.C.B*,**25**, 7520-7530.